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Previews

siRNA: A Guide for RNA Silencing

RNAi is routinely used to eliminate gene activity for experimental purposes. However, the precise molecular mechanism of RNAi is unknown. Recent papers partially illuminate this mechanism in human cells, advancing the potential application of RNAi toward the treatment of human disease.

RNA interference (RNAi) is a form of posttranscriptional control in which the introduction of a double-stranded RNA (dsRNA) into a cell leads to specific degradation of mRNAs with complementary sequence. RNAi was first discovered in *C. elegans* but has subsequently been found in other animal species, including *Drosophila* and humans. Mechanisms of posttranscriptional control similar to RNAi also exist in plants and are known as PTGS (posttranscriptional gene silencing) and cosuppression, and in fungi (quelling) [1]. The natural function of RNAi is thought to be a primitive immune response against parasitization by foreign nucleic acids, such as RNAs from viral pathogens, and rapid spreading of transposons and retroposons. The RNAi molecular machinery is also partially involved in the generation of single-stranded micro-RNAs (miRNAs), some of which are involved in developmental patterning [2, 3].

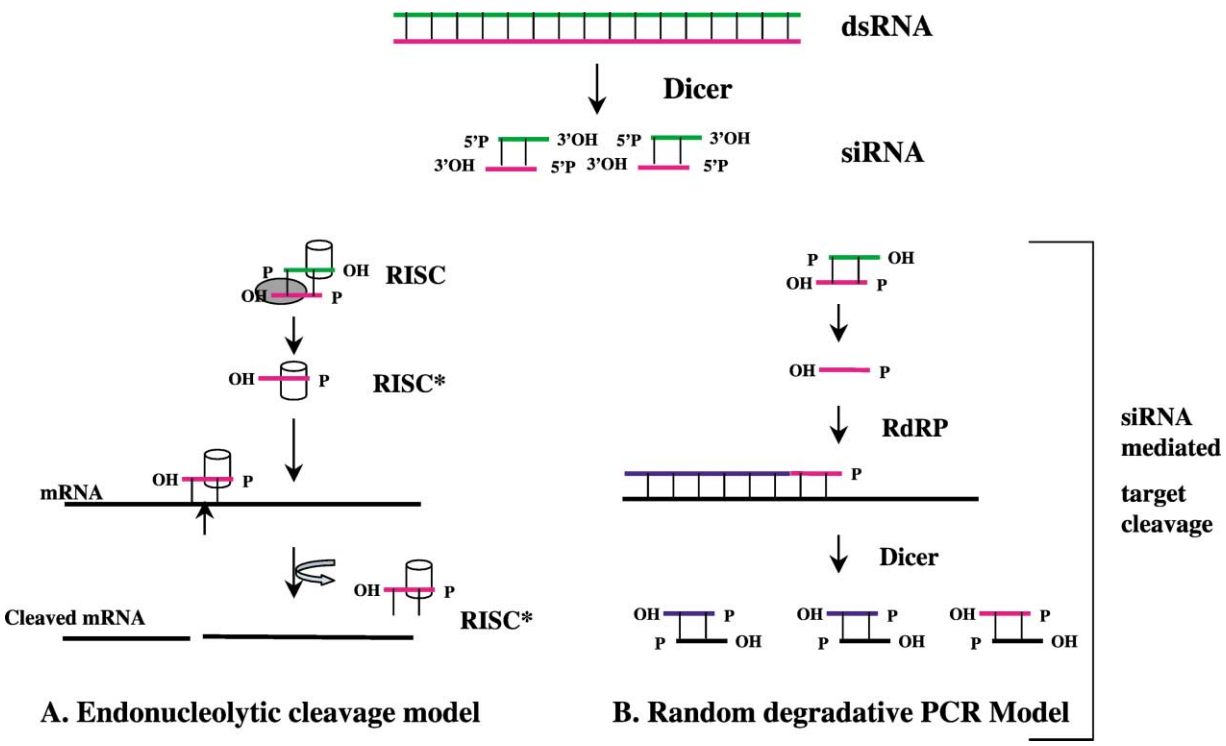
Since its discovery in *C. elegans*, RNAi has been developed as an important tool for reverse genetics. RNAi is now being used routinely to knock down gene function in molecular genetic dissections of biological processes [1]. The existence of RNAi in human cells, and the development of RNAi in cell cultures, suggests the intriguing possibility that this procedure could be developed as a powerful tool for gene therapy in humans [4]. However, despite the widespread use of RNAi as a tool in biological research, the molecular mechanism of RNAi is not understood. Although many of the factors involved in RNAi have been identified through mutation studies or by biochemical purification, the roles of many of these factors remain unclear, and although RNAi and similar phenomena such as PTGS, cosuppression, and quelling share common features, it is not known if they use an identical mechanism.

In vitro studies have defined the initial steps of RNA interference: double-stranded RNA is converted to 21–23 double-stranded nucleotide fragments, called small interfering RNAs (siRNAs), by the RNaseIII enzyme, Dicer [5, 6]. Such RNAs have also been observed in plants undergoing posttranscriptional gene silencing or cosuppression [7]. The mechanism by which these siRNAs mediate the cleavage and destruction of RNA is being actively investigated by several groups. Recent papers from the Zamore [8] and Rana groups [9] make significant advances in shedding light on the mechanism of siRNA-mediated RNA silencing in human cells.

Two competing models of siRNA function have been presented in the literature: the "Random Degradative PCR" model and the "Endonucleolytic Cleavage" model (see Figure). The Random Degradative PCR model proposes that siRNAs act as *primers* on the target mRNA, akin to the primers used for PCR. This model is derived from time course studies [10] by Paterson's group that show that ^{32}P-labeled siRNAs bind to their complementary target mRNA and are extended by nucleotide addition in a target-dependent manner, ultimately converting the single-stranded target mRNA to dsRNA (see Figure, panel B). As this reaction continues, the dsRNA is fragmented, eliminating the target RNA and generating new siRNAs. This Degradative model requires both RNA-dependent RNA polymerase (RdRP) and Dicer to explain the observation of siRNA-mediated target destruction. In support of this, single-stranded antisense RNAs ranging from 19 to 40 nucleotides have been found to be effective in germline RNA silencing in *C. elegans* [11]. Such silencing is dependent on a 3′-OH group and Dicer, indicating both RdRP and Dicer are involved in RNA silencing [11]. Indeed, mutation of RdRP leads to the abolition of posttranscriptional gene silencing in plants [12], quelling in *N. crassa* [13], and RNAi in *C. elegans* [14]. The involvement of RdRP in the Degradative model of siRNA function could also explain the requirement for the presence of a 3′-OH group on siRNA [10], as well as the observation in *C. elegans* of "transitive RNAi," which is the spread of the effect of RNA silencing originating from the 3′ end of the target mRNA toward 5′ end [15].

The Endonucleolytic Cleavage model hypothesizes that siRNAs act as *guides* for proteins to cleave the target mRNA [16]. In this case, the siRNAs combine with proteins to form a riboncleoprotein complex called RISC (RNA-induced silencing complex). Upon activation with ATP, siRNAs are unwound and guide activated RISC (RISC*) to cleave the target. This model is supported by many in vitro studies. Zamore's group showed that cleavage of the target mRNA in *Drosophila* extracts is seen only in the region exactly complementary to the to input siRNA and does not spread to the 5′ end of the target mRNA [16, 6]. In addition, functional studies of siRNA in *Drosophila* lysates have demonstrated that each siRNA duplex cleaves its target RNA at single site [17]. Further, Zamore's group showed that RISC is turned over multiple times, presenting evidence for its catalytic nature [18]. This model does not invoke the involvement of RdRP activity in siRNA-mediated silencing.

The Degradative PCR model predicts a compulsory requirement of a 3′-OH group on guide/antisense strand of siRNA to allow for RdRP activity. Recently published studies provide evidence that blocking the 3′ end of the guide strand with either 2′3′dideoxy cytidine, amino modifier [8], puromycin, or biotin [9, 19] does not inhibit siRNA action either in vivo or in vitro in *Drosophila* and human systems. Therefore, siRNA extension by RdRP does not appear to be a necessary step in siRNA-mediated cleavage/silencing in these systems, despite the clear evidence for such an RdRP activity in *Drosophila*



Schematic Representation of RNAi Pathway Derived from *Drosophila* Extracts

There is consensus regarding the initiation step of RNAi, i.e., that Dicer converts longer dsRNA into 21–23 nt ds siRNA fragments. However, it remains unclear as to how siRNAs mediate the cleavage of target mRNA.

(A) The endonucleolytic cleavage model proposes that siRNAs act as guides. siRNAs form a precursor protein complex called RNA-induced silencing complex (RISC). Upon ATP activation, it unwinds the siRNAs and converts into an activated RISC (RISC*). RISC* recognizes the target and cleaves it with an endonuclease.

(B) In contrast, the random degradative PCR model proposes that siRNAs act as primers on the target mRNA and are extended by RdRP to produce dsRNA (cRNA/target RNA hybrid). The ds cRNA/target RNA hybrid is degraded by Dicer. There is a strict requirement of RdRP and Dicer in this model.

extracts. Since a canonical RdRP homolog has not been found in either the *Drosophila* or human genomes, a noncanonical RdRP may perform this function. However, there is a strict requirement of 5′-phosphate group on the guide strand of the siRNA. A requirement for 5′-phosphorylation of siRNAs is observed both in *Drosophila* and human lysates in vitro [8] and human cell in vivo [9]. Another characteristic of the Degradative model is that Dicer must acts twice in the pathway to eliminate the mRNA (see Figure). However, Tuschl and colleagues [19] have shown that immunodepletion of Dicer from human extracts does not inhibit siRNA mediated cleavage. Also, partial purification of RNA-induced silencing complex from human cells or *Drosophila* cells does not contain Dicer [19]. These results do not support the idea that siRNA-mediated cleavage is dependent on Dicer; however, it has not been determined whether completely eliminating Dicer function in vivo by gene knockout has an effect on siRNA-mediated RNAi in insect and mammalian cells.

It is tempting to speculate that in human cells, RNAi does not function via the Degradative Model because the accumulation of long double-stranded RNA would activate the interferon response pathway, resulting in general inhibition of translation [20], rather than specific posttranscriptional interference of a gene. Therefore, for the Degradative Model to work, Dicer would have to be extremely efficient to avoid steady-state accumulation of dsRNA. In *C. elegans*, on the other hand, the Degradative Model of siRNA function could be tolerated, as the presence of dsRNA is known to result in specific RNA interference rather than general translation inhibition. It is interesting that both the Degradative and Endonucleolytic models are derived from experiments in *Drosophila* embryo extracts. In this system, initial experiments [10] showed that siRNA priming and extension occurred, but in other work [16] it was reported that siRNA leads to cleavage of a target only at one site. How can one reconcile such disparate results? Perhaps RdRP activity is not active in the extracts prepared from Zamore and Tuschl's group, or the enzyme has been inadvertently activated in *Drosophila* extracts prepared by Paterson's group. These conflicting observations could be clarified in part if an RdRP activity could be purified from *Drosophila* or human cells.

An important result which has emerged from recent studies is that single-stranded antisense RNA can also enter the RNAi pathway, albeit less efficiently, than the ds siRNA [19, 8]. Since a 3′-OH group is not a compulsory requirement for RNAi in human cells, transitive RNAi is not likely to be operating here. These new results from studies of human and insect cells encourage and

support the use of siRNAs to knock down splice variants, gene isoforms, and mutants bearing single nucleotide variations. Now, with this information in hand, RNAi may prove even more useful in functional genomic studies and in the treatment of human disease.

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Sensing Nickel: NikRs with Two Pockets

NikR represses expression of a nickel transporter in response to elevated levels of Ni(II). Recent results suggest that repression is elicited by binding of nickel to a high-affinity site, but a low-affinity binding pocket may also play a role.

Metal ions present a conundrum to the cell: they are essential for life, yet they can also be toxic when present in excess. In recent years, considerable progress has been made in defining the pathways by which cells acquire, distribute, store, and export metal ions. Metal ion homeostasis typically involves one or more high-affinity uptake pathways that are induced when metal ions are limiting and storage or efflux mechanisms induced when metals are in surplus. Responsibility for regulating these opposing pathways rests with metalloregulatory proteins that sense the intracellular levels of metal ions. In this issue of *Chemistry and Biology*, Chivers and Sauer report biochemical analyses of one such regulator, NikR, that has two distinct metal binding pockets: a high-affinity and a low-affinity site [1].

NikR is a member of the ribbon-helix-helix family of DNA binding proteins and represses Ni(II) transport when nickel levels in the cell are sufficient [2, 3]. Previously, Chivers and Sauer [4] attempted purification of NikR by loading the protein on a Ni-nitrilotriacetate (NTA) affinity column: a common expedient used for the affinity purification of proteins bearing histidine repeats (His-tagged proteins). Unexpectedly, NikR stripped Ni(II) away from NTA and therefore failed to bind the column. Their subsequent studies revealed two Ni(II) binding sites per NikR monomer: the high-affinity site detected in the metal-chelate chromatography and a second, much weaker site (an estimated $K_d > 10^{-5}$ M). Repressor with Ni(II) bound to both sites interacts with operator DNA with exceptionally high affinity (a K_d of 15 pM) [4].

In the current study, Chivers and Sauer [1] characterize the interaction of Ni(II) with the high-affinity site ($K_d \sim 7$ pM) in the carboxy-terminal domain of NikR, which also contains determinants for tetramer formation. Occupancy of the high-affinity Ni(II) sites is sufficient for operator binding ($K_d \sim 30$ nM). This is a reasonably high-affinity interaction for a gene-specific regulatory protein, but is 1000-fold weaker than the affinity exhibited when both Ni(II) sites are occupied. Since both forms of NikR bind operator DNA, albeit with differences in affinity and extent of binding, it is natural to wonder which form mediates repression of the nickel transport operon in vivo.

To address this question, the authors have measured the intracellular concentration of active NikR as ~125 tetramers per cell (200 nM). Since a single Ni(II) ion in the cytosol represents a concentration of ~1.6 nM, well above the dissociation constant of the high-affinity site, NikR will serve as a high-affinity sink for Ni(II). The au-